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PROPAGATION AND PLUG DELIVERY SYSTEM DEVELOPMENT FOR POLYGALA SENEGA (SENECA SNAKEROOT)

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Propagation and Plug Delivery System Development for *Polygala senega* (Seneca Snakeroot)

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Abstract

Research carried out from 2000 - 2002 for the Agriculture Development Fund project # 19990338 (a continuation of the Agri-Food Innovation Fund project #96000419) was designed to develop three main propagation techniques for *Polygala senega* plants. Techniques investigated include micropropagation of plants *in vitro* (sterile cuttings), seed germination enhancement, and the production of artificial seeds through somatic embryogenesis. General field and greenhouse husbandry research for *P. senega* was also continued to develop a plug delivery system.

Micropropagation cultures were started from *in vitro* germinates. The stems were cut above the callus and placed in modified Hoagland's media. Culture multiplication was rapid and plants were placed in a series of modified rooting media (modified Hoagland's and modified Murashige and Skoog [MS] media). After two months, these plants were transplanted under greenhouse conditions. Results indicated that while many of the plants survived until transplanting, few had roots and all died within 43 days under greenhouse conditions. The second rooting trial used root crown pieces with stems on modified MS media, of which rooting success varied from 30-34% for two treatments. Results from these rooting trials indicate that *P. senega* can be successfully micropropagated *in vitro*. Further research is required in order to increase rooting success *in vitro* and subsequent post-transplant survival.

Seed germination enhancement trials used a stepwise methodical approach across three treatments including 95% sulphuric acid, 95% potassium hydroxide, and ethanol dipping followed by subsequent burning. Following treatments, seeds were bleach sterilised and placed on growth media in petri plates and gradually acclimatised to higher light densities after root/epicotyl development. The germination rates for acid/alkali treatment normally ranged from 30-50%. The ethanol dipping followed by brief exposure to flame yielded the least germination of all treatments at 0.125%. Peroxide and bleach sterilisation treatments typically yielded <15% contamination – usually bacterial as noted by visual observation during transplanting. The 95% sulphuric acid treatment was the most effective for exposing seed embryos as well as yielding 35-64% germination rates. The potassium hydroxide removed the outer wax layer from the seed effectively, though seed coat removal was not successful, and yielded a germination rate of 45-50%. These results were encouraging with respect to germination rates found in nature, which are thought to be less than 10%. Sulphuric acid treatments were also implicated as the best method for producing germplasm for embryogenesis trials.

Trials for the production of artificial seeds through embryogenesis techniques resulted in the successful production and proliferation of callus from mature seed, however, the callus growth rate was extremely low. Preliminary embryogenicity testing was not successful. Further trials are needed on callus produced from other explants.

Results for greenhouse husbandry trials for *P. senega* indicate that PGX (a commercial soiless rooting mix) was the only media used thus far which is capable of supporting significant growth and survival of transplanted germinates. Given the lack of a net-like root system, a promising mixture of BX soil/clay/flax straw was developed and produced a solid plug. Further testing is required to determine its suitability for plant and root growth. If successful, this mixture would need to be tested for field applications. Field husbandry trials for *P. senega* indicated that most mature plants harvested from natural sites survive and continue to grow after being transplanted in a sheltered outdoor setting. Poor survival for *P. senega* plants raised in the greenhouse and transplanted outdoors indicates that further research needs to be done to increase hardiness and plug survival.

1.0 BACKGROUND AND OBJECTIVES

Polygala senega, an herbaceous perennial, is an economically valuable plant because its root extracts can and have been used in extensively in medicinal and nutraceutical applications. (For a detailed review of this plant see Appendices I & II). In the past, large amounts of Canadian *P. senega* roots were harvested from the wild and sold on international markets. These "wild crafting" practices have had a detrimental effect on the natural distribution of this plant. Once found from New Brunswick to Alberta and as far south as Alabama, healthy populations are now confined to certain specific areas of Alberta, Saskatchewan, and Manitoba.

Concerns have been raised within the herb industry regarding unregulated harvesting which may place the remaining *P. senega* colonies in imminent danger of being seriously depleted. Herb Info Canada, an organisation promoting natural herbs and safe care of natural stands, has stated that *P. senega* should be collected with caution. Although not yet officially listed as an endangered species in Canada (Keane, 1998), *P. senega* is officially listed as endangered in several of the continental United States. Given these realities, it is anticipated that wild harvesting of *P. senega* could become illegal in Canada, similar to the case of the wild leek in Quebec and trillium in Ontario. Working toward multiplying the available seed and making plantlets available to the producer would alleviate the pressure on wild *P. senega*, thus saving this precious medicinal plant from extirpation or extinction.

Presently, the marketplace could absorb all the *P. senega* roots available. Additional demands on *P. senega* include anticipated annual increases of the nutraceutical and functional food markets. Declining availability has resulted in increased export prices, which has reduced root exports somewhat due to decreased international purchasing (personal communication, 2000, Mme. Johanne Dion, Trans-Herb, Longueil, Quebec, root exporter to Germany). In general, the industry is faced with a situation where the demand for the product is rising while the supply is declining.

In addition to the functional food and nutraceutical market, *P. senega* roots may become important in the pharmaceutical industry. *P. senega* roots contain saponins that have been identified as Senegin II ($C_{70}H_{104}O_{324}\cdot H_2O$), Senegin III ($C_{69}H_{102}O_{314}\cdot H_20$), and Senegin IV

($C_{75}H_{112}O_{354}\cdot H_2O$), and have been shown to have important expectorant and hypoglycaemic properties. It is reasonable to assume that these molecules may become patented pharmaceutical molecules within the next decade. The University of Saskatchewan has a patent pending for *P. senega* root compounds that contain adjuvant and immuno-stimulating properties (Estrada *et al.*, 1999). Large-scale production of *P. senega* would thus be extremely valuable to both pharmaceutical and veterinary sectors. Developing new protocols for the propagation, cultivation, and processing of *P. senega* may address the rising root demands for this plant without depleting natural stocks.

Challenges for commercial production of *P. senega* include poor germination, seed viability and a plug production system. By producing *P. senega* through various plant biotechnology techniques, domestication of this crop is possible thus creating opportunities for Saskatchewan producers. In addition, the development of these protocols would further enable Saskatchewan producers to diversify their operations by growing a crop that is not as dependent on dry harvest conditions. Harvesting this crop could be done when fields are wet, lending itself to offsetting some of the harvest delays normally experienced in oilseed and cereal harvesting.

2.0 PREVIOUS WORK

This project is a continuation from an Agri-Food Innovation Fund project (# 96000419). *Polygala senega* plants have been previously collected from various locations in Saskatchewan and Manitoba. These plants were transferred into two- or three-gallon pots depending on the size of the plants. The care of the plants, which has provided some insight into their growth habits, has also provided direction for *in vitro* production. A seed source was also located in Saskatchewan from which mature and immature seeds were obtained for germination and somatic embryogenesis development.

All previous attempts at somatic embryogenesis have been unsuccessful. Calli were produced from various plant cuttings and embryos, however, all calli subsequently perished. Through an agreement with Dr. K.N. Kao (National Research Council, - Plant Biotechnology Institute), an unpublished chemical seed treatment was suggested. Applying this chemical treatment was very successful at breaking dormancy, and resulted in successful *in vitro* germination of many *P.*

senega seeds. These plants were transplanted into plug trays, over-wintered, and transplanted after re-emergence. Approximately 50% of these plants survived this treatment, however, neither the plug trays nor a second season of growth after transplanting produced a well-formed plug suitable for sale. Mechanical attempts at seed cracking had a negative effect on germination.

Initial research on nutrient regimes for micropropagation of *P. senega* indicated that standard fertilisers blends did not produce satisfactory results. Micropropagates under these earlier trials presented signs of nutrient stress and subsequently perished. A recipe for a daily-use nutrient solution was obtained during subsequent research during 2000 (Appendix III) and has continued to be more successful than the standard fertiliser regime for micropropagation. This solution has also proved to be successful in greenhouse husbandry trials.

Previous attempts at mechanical seed cracking did not improve germination compared to standard chemical treatments. New treatments for improving germination were carried out during 2001.

3.0 FIELD AND GREENHOUSE HUSBANDRY

The purposes of investigating both field and greenhouse husbandry were to learn how to grow and care for *Polygala. senega* plants and to develop a plug delivery system for future commercial production and sales. Very little is known about how *P. senega* plants grow in the wild, and even less about growing this plant in a greenhouse environment. In an attempt to better understand the growth habit of this plant, the native microenvironment was examined. The soils in native stands varied widely from gravely to clay/loam. *P. seneca* plants were also found in open grassland, along the edges of tree stands after mechanical clearing, on hills or on flat ground. The light regimes varied from full sun to partial shading (1,800 – 1000 $\mu mol/m^2/sec$), however the plant appeared to prefer partial shaded conditions. The soil pH ranged from 6.0 - 8.5.

3.1 Materials and Methods

Polygala senega plants were collected from various locations including Limestone Lake, SK, Grand Rapids, MB, Togo, SK and Shell Lake, SK. Plants were removed from these locations during each of two consecutive years (1997 & 1998) and were placed in either one or two-gallon pots and grown in a greenhouse environment. In 2000, 57 plants were removed from two sites in Kingsley RM # 124 (confidential sites) and 63 plants were removed from two sites in Lake Lenore RM # 399 near St. Brieux, SK. These, along with all surviving plants in pots from 1997/1998, were transplanted into the PPS field plot east of Saskatoon, adjacent to an aspen shelterbelt, with rows running east to west. Soil and plant biomass samples taken from collection sites in RM # 124 were analysed at Enviro-Test Laboratories in Saskatoon for analysis (Table 2.3). These analyses assisted in the later formulation of tissue culture media recipes and a greenhouse fertiliser/nutrient solution.

A total of 327 two-year old *P. senega* seedlings, initially germinated *in vitro* in 1999 and later transplanted into 2 ½" pots, were transplanted to the PPS field plot in July 2000. A thick layer of wood chips was placed between the rows to create furrows to minimise wind damage and to provide partial shading. These plants were also covered with suspended jute material to mimic natural shading.

From the mature plants, immature and mature seeds were collected for somatic embryogenesis studies. All plants were regularly watered but not fertilised. Any surviving plants in the field plot were intended to be source material for future micropropagation and somatic embryogenesis study.

In June 2001, additional plants were again collected from the two sites in Lake Lenore RM # 399 along with a representative soil sample from each site. Site 1 was along a road allowance (sandy-loam) while site 2 was in a grassy area (loam) that had been cleared about ten years previous. Ten plants were taken from site 1 and fifty from site 2. These plants were transplanted into the PPS field plot. At this point in time, two soil samples were also taken from the PPS field plot. The four soil samples were then analysed at Enviro-Test Laboratories. By early summer, the suspended jute was replaced with square flax bales placed end to end on the south side of

each row (~6" away from the plant rows). In October, 36 plants were removed from the above mentioned sites in Lake Lenore RM #399, including twenty from site 1 and sixteen from site 2, and subsequently transplanted into the PPS field plot.

For greenhouse trials, germinates produced through seed germination enhancement experiments (see Section 5) were transplanted into various media, including Oasis wedge trays, various plug trays and open flat trays with PGX soil, 1 ¾" square Jiffy poly-fibre peat pots filled with PGX soil, and 1" and 2" Jiffy peat pellets. Various soil types were used, including BX/coconut fibre/PGX combinations. Plants were given water or modified Hoagland's solution daily (Hoagland and Arnon, 1950, Appendix III). Some of the plants were initially given a 10-52-10 fertiliser twice weekly for three weeks to promote root development, after which they were treated as above. Non-germinated seeds were removed from the petri plates and sown into 98-cell tray containing PGX soil. In later trials, non-germinated seeds from a petri plate were placed into one tray cell to save space and to keep each replicate sample together for germination monitoring. A grey shade cloth tent was installed over the germinates in the greenhouse to reduce light intensity. At the end of the growing season, all germinates were examined. Plants with living roots were transplanted into 2 ½" pots and placed into a greenhouse set up for holding dormant plants (average temperature is 0 - $^{+}$3 °C). Plants having dead or dying roots were discarded.

P. senega plants produce a wiry tap root with few lateral roots growing off the main tap root. Previous attempts were unsuccessful at producing a plant that held the soil together with its roots (i.e. bedding plants). The next logical step was to produce a sellable plug that would hold itself together, thus enabling individual plants to be mass-produced and easily removed for shipping and field planting. Three wet transplanting mixtures were prepared to test for suitable plug production in 240-hole styroblocks. Trial #1 consisted of 25% clay, 25% coco fibre and 50% BX soil. Trial #2 consisted of 25% clay, 25% flax straw and 50% BX soil. Trial #3 consisted of 50% clay and 50% BX soil. The mixtures were evaluated after they dried out.

3.2 Results

The soil samples from St. Brieux (SB) and the Prairie Plant Systems (PPS) field plots showed different profiles (Appendix V). The SB1 sample was sandy-loam (roadside) which contained higher levels of all measured elements, especially calcium, in comparison to the two Kingsley RM samples taken in 2000. The SB2 sample (loam - grassy area cleared ~10 years ago) showed element levels closer to the Kingsley samples. The two samples from the PPS field plot (clay-loam) had lower pH levels compared to all samples tested, in addition to having levels of phosphorus, potassium, sulphur/sulphate, iron and zinc that were many times higher compared to the soil the plants were removed from. The high level of nitrates in the PPS2 sample is not explainable. Soil tests will be repeated to verify these results.

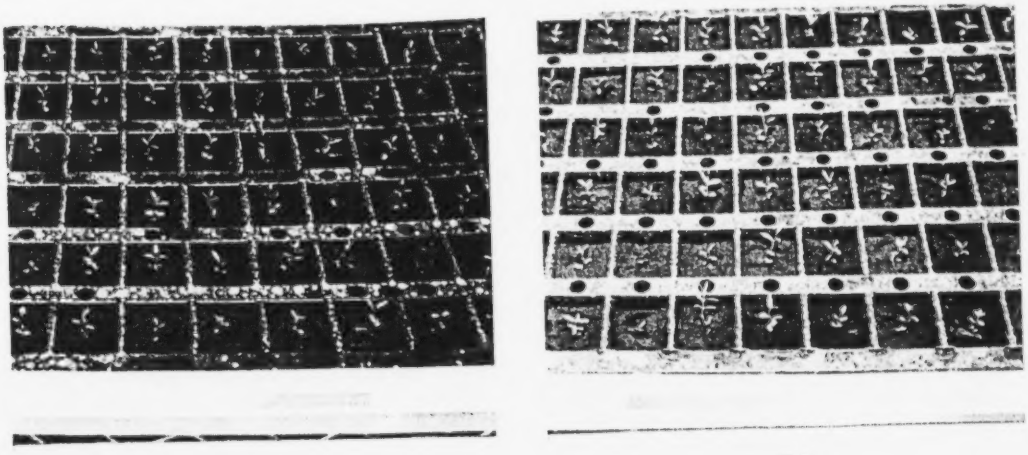
The majority of mature plants transplanted to the field plot in 2000 survived the winter. However, very few (<10/327 or approximately 3%) of the two-year old plants grown in 2 ½" pots survived into the 2001 growing season (Plate 5.1). Plants that were shaded from the south by the flax straw bales appeared healthier. The bales appeared to provide effective shading and protection from the wind. Survival rates of the 2001 transplants will be assessed during 2002.

Plate 5.1. Transplanted seedling with buds (purple) for next year's growth.

For the greenhouse trials, trays of transplanted germinates were covered with a clear dome lid to maintain elevated humidity levels. At approximately two weeks, the plants were acclimatised by slowly removing the domes over the period of one week. Plant survival was estimated at 42%

over the course of the experiment across all treatments, with substantial variation between treatments. Actual survival rates were difficult to determine due to the plant's tendency to go into early dormancy. Survival rates will be determined in the spring of 2003 when the transplanted germinates come out of dormancy.

The Oasis foam plug, which looked promising when it was initially used in November/December 2000 (Plates 5.2 a and b), proved to be a poor growth medium for the germinates. Within two months, the plants looked stressed; the leaves turned purple and roots curled around inside the planting hole and turned yellow (initially white). Plants placed into this medium were compromised due to the inability of the roots to penetrate the foam, and from the tendency of the foam to retain too much water.



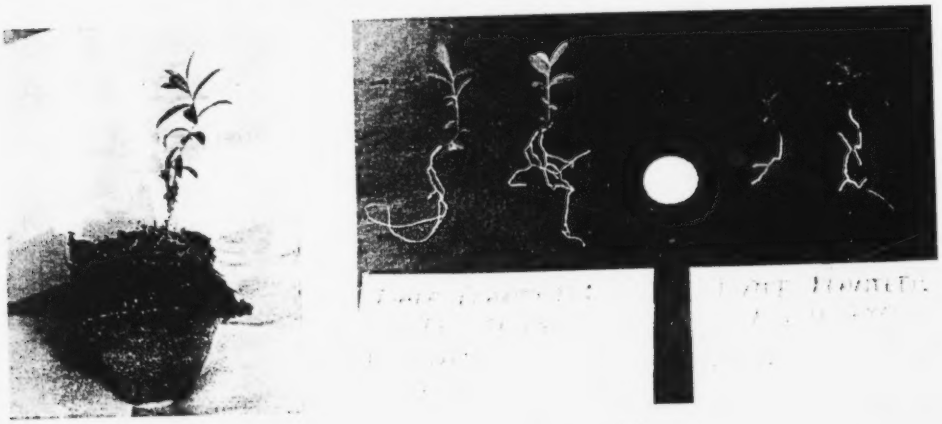
(a) (b)

Plates 5.2 a and b. Good growing germinates in Oasis foam plugs.

The 2" netted Jiffy peat pellets were also not a successful media for *P. seneca* germinates. Similar to the Oasis foam, the latter peat pellets tended to hold large amounts of water to the detriment of the germinates, especially when used with a solid bottom container that did not allow water drainage. The 1" Jiffy peat pellets without nets were also unsatisfactory, as they tended to fall apart when watered.

Germinates planted into PGX soil thrived compared to those planted in the Oasis foam or BX/coconut fibre/PGX blends. Most of the seneca plants placed into the PGX soil grew well

and showed excellent root development (Plates 3.1a,b). There was no visual difference between plants grown in the variety of pots used containing PGX soil (Jiffy peat pots, 98-round hole trays, 2 ½" plastic pots). Epicotyl and root growth on all these plants were very similar.



a) b)

Plate 3.1. a) Germinate in peat pot with PGX soil at end of growing season. Note new buds at root crown.
b) Growth comparison between germinates in PGX soil and Oasis foam.

Any plants growing in open flats were later transplanted into either 98-hole trays or the square Jiffy peat pots with PGX soil (Plate 3.2). It was anticipated that these peat pots would be suitable for both growing and transporting seneca plugs. However, by the end of the growing season, the peat pots were decomposing and the plants had to be placed into plastic 2½" pots. Overall, the 2 ½" plastic pots allowed for the greatest root development.

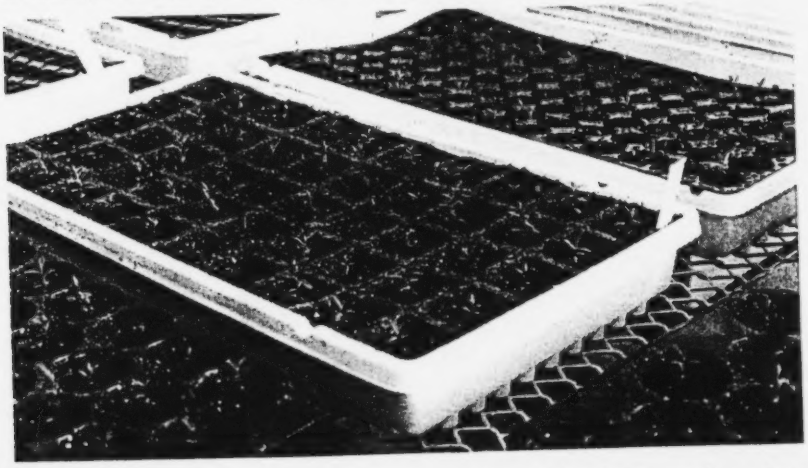


Plate 3.2. Germinates growing in various plug trays and peat pots.

The flax straw/clay/BX mixture in plug production trial #2 caused challenges in filling the styroblock holes while trial mixtures #1 (clay/coco fibre/BX) and #3 (clay/BX) easily filled the styroblocks. However, after the styroblocks were allowed sufficient drying time (> 1 week) and the test plugs removed, trial #2 produced the only plug which held together and was the easiest to remove of the three trials.

3.3 Discussion

P. senega germinates are slow growing, and root development does not hold soil together -- even after two growing seasons. The root starts off as a taproot that eventually branches out, but it does not form a net of roots needed to create a firm, self-contained plug. Therefore, in order to produce *P. senega* plugs, the plug container would need to be self-supporting with minimal breakdown during production. The container, however, would have to be biodegradable upon field planting. Oasis foam was thought to be ideal as it is pH neutral, does not lose its shape and can hold water efficiently (up to 40 times its weight) to ensure seedlings do not desiccate. The Oasis foam ultimately proved to be detrimental to seedling survival due to an inability of the roots to penetrate the foam in addition to a tendency of the foam to retain excess water. Jiffy plugs with netting should be repeated on a larger scale (>300) using draining trays. Viable plants in 2 ½" pots will be grown for at least one more year before being transplanted into the field plot to allow for sufficient root development and a greater chance of survival.

Plants in the field plots grew better when adequate protection from the wind was introduced in the form of rectangular flax bales placed adjacent to the rows of seneca. The bales also produced more natural shading than the suspended jute material previously used. Excessive outdoor losses of transplanted germinates from previous greenhouse trials may have been due to excess compaction from the wood chip mulch, snow cover and the effects of wind. The wood chips were initially raised on each side of the rows with a light covering around each plant. By the next spring, it was difficult to identify some of the rows due to redistribution of the chips.

The clay/BX/flax plug mixture may be improved by changing the ratio of ingredients to include more clay for a stronger plug. The mixture's pH could be modified to mimic natural seneca soil

conditions with crushed lime. This mixture needs to be tested with germinates to determine whether it sustains plant health and allows for suitable root growth throughout the plug.

3.4 Conclusion

Despite challenges related to the propagation and survival of *P. seneca* under greenhouse and field conditions, several milestones were achieved. While germinate survival under both greenhouse and especially field conditions has been lower than anticipated, the discovery of a successful plug mixture indicates that the development of a deliverable plug system is nearly complete. Further tests should determine whether this mixture sustains plant growth. Field husbandry trials for *P. senega* indicated that most mature plants harvested from natural sites survive and continue to grow after being transplanted in a sheltered outdoor setting.

4.0 MICROPROPAGATION

The purpose of the micropropagation study was to produce *P. senega* plantlets *in vitro*, eventually achieving rooted plants that can be transplanted in a greenhouse for further growth. Micropropagation is the asexual propagation of plants from growing tissue -- be it whole plants or established callus culture. Normally, stem cuttings are further cut into pieces, ensuring each contains at least one dormant lateral bud that can flush and grow into an identical copy of the parent plant when the apical dominance of a plant is disrupted. Callus (indeterminate and unorganised cellular growth produced mainly during somatic embryogenesis) can also be induced to differentiate and grow into shoots. Callus growth also occurs at the bottom of the explant, which can differentiate into bud-like entities and produce copies of the original plant. In time it is possible to produce 10+ plants from each cutting initiated. The process is controlled through the use of a relative high cytokinin level (such as benzylaminopurine - BAP) and a low relative auxin level (such as indole-3-butyric acid – IBA, α-naphthaleneacetic acid – NAA or 2,4-dichlorophenoxyacetic acid – 2,4-D). As micropropagated plants are typically shorter and smaller than those grown naturally, giberillic acid (GA_3) can be added to increase the internodal and overall height. Since very little is known about *P. senega* nutrient requirements, a propagation media was prepared based on the Hoagland's nutrient solution for micropropagating germinates.

4.1 Materials and Methods

Growing cultures were originally produced by transferring *in vitro* germinated seeds into small glass jars containing a modified Hoagland's nutrient solution (pH 6) supplemented with 6% sucrose, BAP or 2,4-D and 0.6% Phytagar. This media was soon revised (Appendix III) for macro- and micronutrients. 0.1 mg/l 2,4-D (4 µM) was used initially along with 7.5 mg/l BAP. Sufficient multiplication and growth was later found using 0.1 mg/l IBA with 2.5 mg/l BAP.

After a sufficient number of germinates had been produced, a rooting trial was undertaken using ½ strength modified Hoagland's and ½ strength Murashige and Skoog media (MS, Appendix IV, Murashige and Skoog, 1962), each containing 1.5, 1.75 or 2 mg/L IBA with and without 2 mg/L NAA. Individual stems were cut off above the callus and placed in the media, nine per jar. Two months later, all plants with roots along with 85 plants without roots were transplanted at the greenhouse. The rooted plants were placed into 2½" pots while the non-rooted plants were cut on the bottom of the callus, dipped into #4 rooting powder (0.4% IBA) and placed into a 162 x 20ml chamber tray. The plants were then placed into a mist tent (~70% humidity) for two weeks after which they were slowly acclimatised to a natural environment.

A second rooting trial was later performed using ½ MS media containing either 1.5 or 2.5 mg/L IBA with 0.4 mg/L NAA. For this trial, the root crown was divided into 3-4 pieces, each containing 1-4 long (4-5 cm) stems. Since a few of the cultures already had roots, the crowns were divided (while keeping the roots intact) and transferred to rooting media.. As well, the bottom of the root crown was cut horizontally to expose the inner crown cells. It was believed that since all roots and stems emerge from the root crown in nature, root crown cells might hold the ability to form roots.

4.2 Results

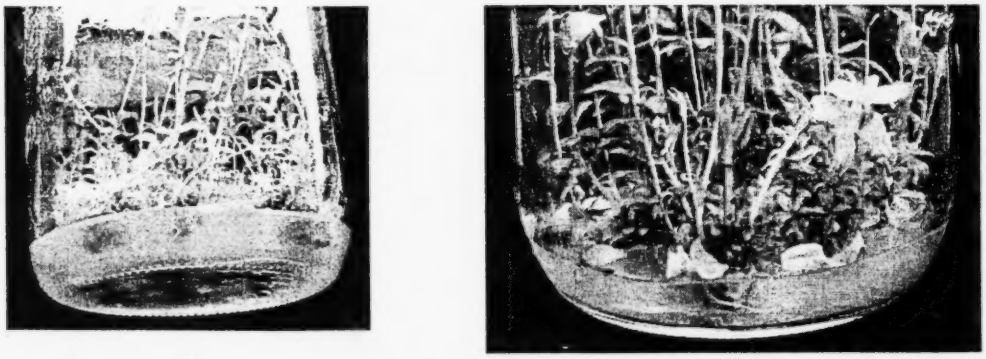
P. senega plants grew well in culture and was observed multiplying 10-15X when left for six weeks between subcultures (Plates 4.1 a and b). However, within this propagation time frame, there was a loss of ~10-20% of plants. Poor growing plants appeared stressed, and turned deep purple before dying. Good growing plant clumps had multiple stems, some of which lost their

colour and died but the overall clump multiplied well. Individual stem or plant mortality did not appear to affect other plantlets in the jar.

(a) (b)

Plates 4.1 a and b. Micropropagated *P. senega* plants on modified Hoagland's media.

The first micropropagation rooting trial was inconclusive, though some of the treatments did accomplish rooting *in vitro* (Plate 4.2). Because the plants were separated from the callus (equivalent to the root crown in native plants), only 32.73% survived to be transplanted under greenhouse conditions (Table 4.1). The transplants without roots died within two weeks. All plants in the first rooting trial transplanted at the greenhouse died within 43 days, even though they received the same Hoagland's nutrient solution that the transplanted germinates received.



(a) (b)

Plate 4.2. Micropropagated plants showing various amounts of root formation.

The second rooting trial showed a marked improvement. The media containing 1.5 mg/l IBA had 34.4% rooting while the media with 2.5 mg/l IBA had 30.0% rooting. *In vitro* plant death ranged from 20-26%, a drop of 40-45% over the first trial. Plants with either roots or no roots were shipped to the PPS greenhouse and were transplanted. Survival rate was high for both as many of the plants without roots rooted readily in soil.

Table 4.1. Initial *in vitro* rooting trial results

Media**	Alive - roots	Alive - no roots	Dead - roots	Dead - no roots
HG/1.5/0	0	88	0	92
HG/1.75/0	0	63	0	117
HG/2/0	0	70	0	110
HG/1.5/2	0	47	0	133
HG/1.75/2	0	39	0	141
HG/2/2	0	63	0	117
TOTAL	**0**	**370/1080=34.26%**	**0**	**710/1080=65.74**
MS/1.5/0	0	70	0	110
MS/1.75/0	0	36	0	144
MS/2/0	4	99	0	77
MS/1.5/2	2	18	0	160
MS/1.75/2	0	28	0	152
MS/2/2	10	70	0	100
TOTAL	**16/1080=1.48%**	**321/1080=29.72%**	**0**	**743/1080=68.80%**

**Note: Media designation: HG: ½ Hoagland's Media, MS: ½ Murashige Skoog. The first number after media represents IBA level (mg/L) and second number represents NAA level (mg/L).

4.3 Discussion

While the modified MS media has shown some promise for producing roots *in vitro* in comparison to the modified Hoagland's media, further research needs to be done to optimise *in vitro* rooting. The overall rooting success may have been limited by a less than ideal combination of nutrients. For instance, it is possible that nitrogen levels in the media used may be too high. It has been suggested in various commercial nursery catalogue publications that nitrogen inhibits phosphorus intake in plants and that the P:Fe ratio is critical for plant growth (Professional Gardener, 1994; Westgro, 2001). Phosphorous is important for root growth in general, and it is known that phosphorus is found in high concentrations in a plant's seeds and roots (50-100+ ppm). As seen in Table 4.2, the Hoagland's and MS rooting media are very similar in micronutrients but very different in potassium, sulphate, calcium, magnesium and iron.

The Hoagland's medium has a P:Fe ratio of 2.01:1 compared to 7.48:1 in the MS medium. It cannot be determined at this time if the reason for the rooting in MS is due to the differing nutrient levels. As well, the IBA/NAA treatments were equal for both media and the plants were prepared using the same methods for all treatments. A propagation media that encourages root production at each step would be highly beneficial as it would eliminate one step in the process.

Table 4.2. Nutrient comparison between modified Hoagland's propagation and rooting and modified MS rooting media

Nutrient	Hoagland's Propagation (ppm)	Modified Hoagland's Rooting (ppm)	Modified MS Rooting (ppm)
Ammonia (NH_4)	---	---	825
Nitrate (NO_3)	3200	2400	1775
Phosphate (PO_4)	142	107	85
Potassium (K)	950	603	1039
Sulphate (SO_4)	538	404	198
Calcium (Ca)	1200	900	0.21
Magnesium (Mg)	520	390	185
Iron (Fe)	400	300	139
Boron (B)	3.5	2.6	3.1
Manganese (Mn)	13.6	10.2	8.4
Copper (Cu)	2.8	2.1	4.3
Zinc (Zn)	1.5	1.2	0.13
Molybdate (MoO4)	0.24	0.18	0.14
Cobalt (Co)	---	---	0.013
Iodine (I)	4	3	2

4.4 Conclusion

P. senega can be successfully micropropagated *in vitro* using either a modified Hoagland's or modified MS media. The latter MS media also appears to have some potential of encouraging minimal root production. Further research needs to be done to encourage higher rates of rooting production, which may improve post-transplant survival rates.

5.0 SEED GERMINATION ENHANCEMENT

5.1 Introduction

When it became evident that somatic embryogenesis research was not yet producing the desired results, another approach for producing plants to be transferred to the greenhouse was initiated.

The seeds are small (3/16" long, ~585 per gram, Plate 5.1) at maturity and have a hard seed coat that is covered with a waxy coat that hinders water absorption. Because of this, chemical or bacterial degradation of this outer coating is required before germination can occur. In nature, *P senega* germination is extremely low (estimated at <10%). A

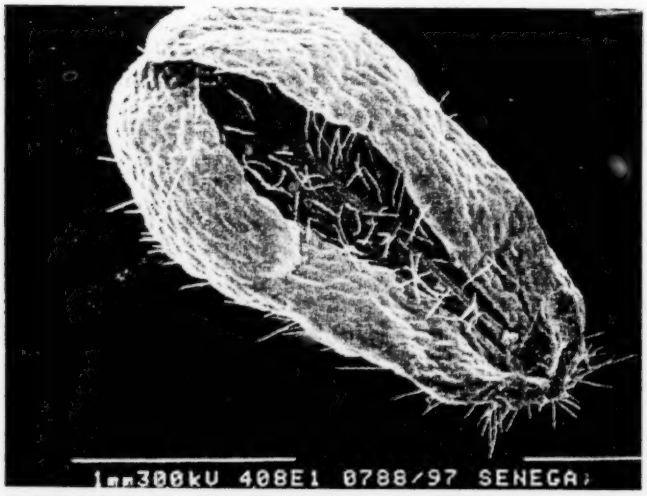


Plate 5.1. *Polygala senega* seed magnified 30 X.

number of treatments were devised to enhance the germination success of the seeds, using a stepwise methodical approach, including immersing seeds in two different chemicals for different time periods - 95% sulphuric acid and 95% potassium hydroxide. Sterilisation efficiency using 10% hydrogen peroxide and 10% bleach was also tested.. Ethanol dipping followed by subsequent burning was also attempted to remove the waxy outer shell. Following treatments, seeds were placed on growth media in petri plates and gradually acclimatised to higher light densities. A treatment involving liquid nitrogen was also considered but was ultimately not incorporated into the experimental design. Germinated seeds from these treatments were shipped to the greenhouse to provide stock plants for future use and to be used in transplant trials.

5.2. Materials and Methods

An initial sterilising trial using 10% bleach (5.25% sodium hypochlorite) for 5-8 minutes was repeated. The corresponding 10% hydrogen peroxide test had only one replicate of a 5-minute

test performed at this time. Seed was initially treated for seven minutes with 95% sulphuric acid, rinsed with sterile water for three minutes, followed by the sterilisation test and 3 X 3 minutes sterile water rinses. All seeds were then placed onto rooting media in petri plates (20 per). All germinated seed was transplanted at the greenhouse for husbandry testing. Two subsequent trials were completed. The bleach trial was repeated three times while the peroxide trial was repeated twice.

Another trial was undertaken comparing 95% sulphuric acid and an equimolar strength potassium hydroxide (KOH). The sulphuric acid is approximately 18 M. Using 85% KOH pellets, an equal strength solution needed to be 20.1 M. A 2:1 ratio of these solutions were tested on seeds for 3-8 minutes to determine which method was best to remove the seed coat and at what length of time provides the highest germination without killing the seeds. The KOH solution solidified at room temperature and required heating on a hot plate. The seeds were treated with ~80°C KOH for the various test times. A drop of Tween 20 was added to the KOH to break the surface tension. The seeds and KOH were gently agitated with a spoonula. The seeds were then transferred to a sterile tea ball and rinsed for 3 minutes in sterile water, 7 minutes in 10% bleach followed by 3 X 3 minute sterile water rinses. The KOH-treated seeds were vacuum-filtered onto sterile filter paper and placed into petri plates (20 per) containing rooting media. The sulphuric acid-treated seeds were manually placed into petri plates (20 per) containing rooting media covered with a sterile filter paper. The plates were placed in the dark for two weeks followed by gradual acclimatisation to higher light intensities. Once the epicotyl emerged, the seeds were sent to the greenhouse. The acid trial was repeated three times while the alkali trial was repeated twice.

The ethanol/fire trial was accomplished by placing the seed sample in a teaball, immersing it in 95% ethanol to coat the seeds and then allowing the excess liquid to run off. Then the tea ball was passed through a flame, rotated and shook slightly to ensure all the ethanol burned off. The seeds were then sterilised for 6 minutes in bleach and placed on rooting media as above. Because of the poor initial results, this trial was not repeated.

5.3. Results

Every trial sent to the greenhouse was checked for bacterial or fungal contamination and recorded. Each trial had 13-15 petri plates with three replicates individually assessed for germination rates and presence of contamination. Plate 5.1 shows seeds germinating on ½ MS. The pooled results for the three replicates of each timed trial for sterilisation and seed cracking are presented in Tables 5.1 and 5.2.

Plate 5.1: Germinated seeds after one month.

Table 5.1. 2001 Contamination and germination study results comparing between sterilisation and seed cracking trials

Trial	Time	# PP	#PP-fungus/%	#PP-bacteria/%	# Germ	% Germ
10% peroxide	5 min	43	1 / 2.3	4 / 9.3	372	44.8
10% peroxide	6 min	44	0 / 0	5 / 11.4	356	42.6
10% peroxide	7 min	42	0 / 0	2 / 4.8	436	53.8
10% peroxide	8 min	42	0 / 0	7 / 16.7	398	48.1
10% bleach	5 min	42	0 / 0	6 / 14.3	465	56.8
10% bleach	6 min	42	0 / 0	3 / 7.1	431	52.8
10% bleach	7 min	41	1 / 2.4	5 / 12.2	425	53.1
10% bleach	8 min	42	1 / 2.4	8 / 19.0	475	58.3
H_2SO_4	3 min	43	0 / 0	2 / 4.7	380	44.6
H_2SO_4	4 min	43	0 / 0	4 / 9.3	379	49.5
H_2SO_4	5 min	42	0 / 0	4 / 9.5	430	53.1
H_2SO_4	6 min	42	1 / 2.4	3 / 7.1	334	41.6
H_2SO_4	7 min	43	0 / 0	4 / 9.3	301	36.3
H_2SO_4	8 min	41	0 / 0	5 / 12.2	399	49.1
Ethanol/heat	2 X	43	2 / 4.7	7 / 16.3	2	0.2
Ethanol/heat	3 X	44	3 / 6.8	5 / 11.4	1	0.1
Ethanol/heat	4 X	44	0 / 0	5 / 11.1	1	0.1
Ethanol/heat	5 X	44	1 / 2.3	4 / 9.1	1	0.1
10% bleach	5 min	42	1 / 2.4	7 / 16.7	309	38.1

Table 5.1 continued.

10% bleach	6 min	42	1 / 2.4	7 / 16.7	305	38.2
10% bleach	7 min	41	0 / 0	3 / 7.3	370	45.8
10% bleach	8 min	42	1 / 2.4	5 / 11.9	207	25.7
H_2SO_4	3 min	57	1 / 1.8	2 / 3.5	485	43.1
H_2SO_4	4 min	42	0 / 0	0 / 0	316	38.7
H_2SO_4	5 min	42	0 / 0	0 / 0	339	41.0
H_2SO_4	6 min	40	0 / 0	2 / 5.0	496	64.1
H_2SO_4	7 min	42	0 / 0	2 / 4.8	311	38.0
H_2SO_4	8 min	42	0 / 0	2 / 4.8	220	27.2
KOH	3 min	42	3 / 7.1	4 / 9.5	279	33.4
KOH	4 min	42	3 / 7.1	8 / 19.0	239	28.5
KOH	5 min	42	3 / 7.1	4 / 9.5	463	54.5
KOH	6 min	44	2 / 4.5	8 / 18.2	368	43.5
KOH	7 min	45	2 / 4.4	12 / 26.7	311	35.5
KOH	8 min	42	2 / 4.8	7 / 16.7	417	48.0

Table 5.2. 2002 Contamination and germination comparing between sterilisation and seed cracking trials.

Trial	Time	# PP	#PP-fungus/%	#PP-bacteria/%	# Germ	% Germ
10% peroxide	5 min	43	4 / 9.3	2 / 4.7	232	27.3
10% peroxide	6 min	42	2 / 4.8	3 / 7.1	286	35.1
10% peroxide	7 min	43	0 / 0	3 / 7.0	144	17.3
10% peroxide	8 min	41	0 / 0	2 / 4.9	206	26.0
10% bleach	5 min	44	1 / 2.3	0 / 0	174	20.0
10% bleach	6 min	41	0 / 0	3 / 7.3	248	30.6
10% bleach	7 min	43	2 / 4.7	1 / 2.3	214	25.5
10% bleach	8 min	42	1 / 2.4	5 / 11.9	232	28.6
H_2SO_4	3 min	42	2 / 4.8	1 / 2.4	74	8.9
H_2SO_4	4 min	43	1 / 2.3	7 / 16.3	151	18.0
H_2SO_4	5 min	41	1 / 2.4	4 / 9.8	208	25.6
H_2SO_4	6 min	43	1 / 2.3	4 / 9.3	282	34.7
H_2SO_4	7 min	40	0 / 0	3 / 7.5	247	31.8
H_2SO_4	8 min	41	0 / 0	1 / 2.4	166	20.6
KOH	3 min	43	3 / 7.0	11 / 25.6	2	0.2
KOH	4 min	43	1 / 2.3	7 / 16.3	8	0.9
KOH	5 min	43	0 / 0	6 / 14.0	17	2.0
KOH	6 min	45	1 / 2.2	6 / 13.3	22	2.6
KOH	7 min	44	0 / 0	9 / 20.5	14	1.7
KOH	8 min	43	3 / 7.0	12 / 27.9	16	1.9

The plants were shipped to the PPS greenhouse one month after the trials. However, the greenhouse survival rate is unknown at this time as all the plants are dormant.

5.4. Discussion

The sulphuric acid appeared to provide the best results for cracking the seeds out while also acting as a primary surface sterilant. The seeds endured neither extreme heat nor extreme cold during this treatment. The acid's efficiency decreased when a large aliquot (~30 ml) was removed from the bottle for use (stored in a baby food jar with a snap on lid). The potassium hydroxide was able to remove the waxy coating from the seed, thus enhancing seed germination. However, the acid treatment produced a higher average germination rate compared to the KOH. The sulphuric acid also was associated with relatively lower contamination levels (Tables 5.1 and 5.2). The heating of the seeds during KOH treatment for the 5-8 minutes may have reduced the seed viability.

The chemical and seed mixtures were not heavily agitated during the treatment. The main purpose of the incorporation of chemicals was to remove the outer waxy coating. The seeds were slowly moved back and forth through the chemical with a spoonula. It was desired to have the seed coat itself remain intact to minimise chemical damage to the endosperm and embryo. The seed coat was mainly removed during water rinsing and sterilisation by quick manual agitations of the tea ball. It is believed that the sulphuric acid began to break down the seed coat prior to rinsing in comparison to the potassium hydroxide. As a result, more naked seeds were produced when using sulphuric acid.

Seeds sterilised with peroxide had relatively low contamination rates; however, peroxide treated seeds also had slightly lower overall germination rates in compared to the bleach treatments. Peroxide may be more harmful to the seeds than bleach during sterilisation. Peroxide is known to be detrimental to living cells by producing free radicals. As peroxide is used in a solution, it also breaks down to water and oxygen. Thus, its efficacy decreases with time.

The least effective treatment was the ethanol/heat treatment, which yielded 0.125% germination. It is highly likely that the heat generated essentially 'cooked' the seeds, rendering any essential

germination enzymes useless. The liquid nitrogen trial was not attempted. Through communication with Dr. K.N. Kao (National Research Council - Plant Biotechnology Institute), it was discovered that seneca seed cracking trials with liquid nitrogen had been performed with no resulting germination (unpublished results). It was initially believed that the seeds could not withstand the low temperature of liquid nitrogen immediately coupled with rapid warming in water.

5.5. Conclusion

Results for seed germination enhancement were encouraging. The sulphuric acid and potassium hydroxide treatments have the potential to produce ~ 50+% germination. These germination rates compare favourably to germination rates found in nature, which are believed to be less than 10%. The sulphuric acid worked best at enhancing germination because the embryos were not damaged as much as with the potassium hydroxide. The potassium hydroxide did work, though the solution temperature also needs to be closely monitored. Both the bleach and peroxide were effective sterilants, though the bleach solution was likely less harmful to the embryos.

6.0 SOMATIC EMBRYOGENESIS

6.1 Introduction

Somatic embryogenesis is the asexual production of genetically identical embryos using tissue culture techniques and the manipulation of the embryogenic material using plant growth media containing a high relative auxin level combined with a low relative cytokinin level. When attempting to produce high volumes of cloned plants, somatic embryogenesis is the optimum method since multiplication rates are many times higher than micropropagation. The production process generally contains the following five steps:

- initiation of seed explants *in vitro,* usually from indirectly determined embryogenic cells (Evans *et al.*, 1981)
- induction of embryogenic calli
- proliferation of embryogenic calli
- induction of somatic embryo formation
- maturation of the somatic embryos

Simply put, callus is an undifferentiated growth of cells. In somatic embryogenesis, this growth occurs on the embryo. By incorporating auxins and cytokinins in the media, this growth can be controlled and accelerated. The calli can be subsequently subcultured onto solid media or placed into liquid media. Embryos are produced when the callus is subcultured on an auxin-free media, typically containing abscisic acid (ABA). Mature embryos can be stored and later germinated on an appropriate media. If the callus is not embryogenic, it can still be induced with the aid of different hormones to produce shoots and/or roots.

6.2. Materials and Methods

Naked seeds were obtained by treating seed with 95% sulphuric acid for 10 minutes followed by a two minute sterile water rinse, 7 minutes sterilisation in a 10% bleach (+ 0.1% Tween 20) solution and 3 X 3 minute sterile water rinses. The seeds were resuspended in ~300 ml sterile water with ~30 seed aliquots vacuum-filtered onto 70 mm Ahlstrom #601 filters and placed onto ½ modified MS germination media. Naked seeds were aseptically removed from the filter papers and placed singly into 60 X 15 mm petri plates and 12-well culture plates containing MS or B5 media (Gamborg *et al.*, 1968) to which was added varying 2,4-D and BAP levels (Table 6.1). Some embryos were also removed from the endosperm and placed on the medium. The 12-well plates were wrapped four times with Nescofilm (Karlan Research Products Corp.). Six 12-well plates were stacked, wrapped with aluminium foil and incubated at room temperature. Petri plates were wrapped with Nescofilm, placed three-high in a black tray and incubated at room temperature in the dark. Cultures were observed weekly for callusing. Because of limited growth, any developing callus was not transferred to fresh media for three months.

It was noticed that the majority of the callus growth occurred on B5 media. Fresh B5 media was made with the hormone combinations on which callus was formed. The embryos were given a trial number (1-) and a line number from 1000-1139 (though only 41 embryos callused). The lines that callused were transferred to their fresh respective media. Since only 3 embryos callused on MS media, these were transferred to B5 media:

- 1-1146 was transferred from 2.3 µM 2,4-D/2 µM BAP to 11.3 µM 2,4-D/2 µM BAP (embryos on higher 2,4-D levels produced softer callus than lower levels)
- 1-1168/1-1178 were transferred to B5 media containing the same hormone concentrations.

Table 6.1 . Hormone mixtures used for somatic embryogenesis.

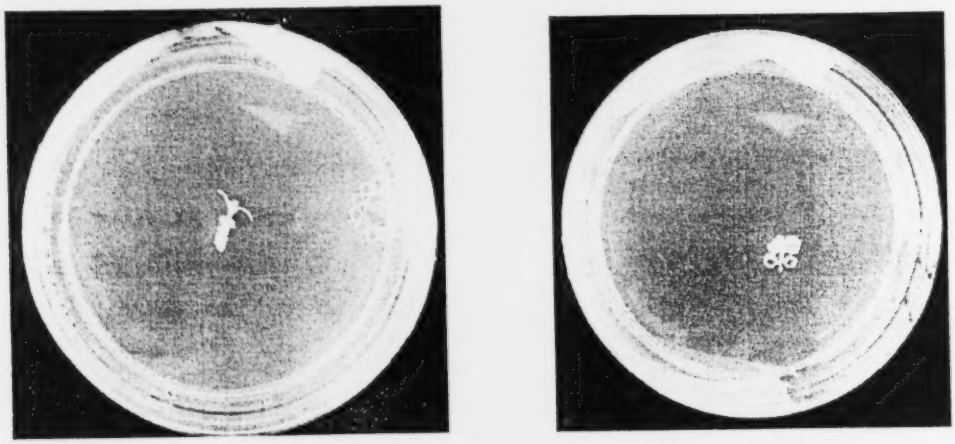
Media							
MS & B5	2,4-D (µM)	2.3	4.5	6.8	9.0	11.3	13.6
	BAP (µM)	2.0	2.0	2.0	2.0	2.0	2.0
	# embryos	36 & 36	36 & 36	36 & 36	36 & 36	36 & 36	36 & 36
MS	2,4-D (µM)	2.3	4.5	6.8	9.0	11.3	
	BAP (µM)	0.21/0.62	0.21/0.62	0.21/0.62	0.21/0.62	0.21/0.62	
	# embryos	43/42	48/44	34/45	51/47	44/54	

The calli were transferred every 45 days (when suitable growth had occurred) onto fresh media until enough callus was formed with which to begin maturation studies (Spruce callus grows quickly and needs to be transferred weekly, personal work experience, 1995-1999). The propagation media was modified at the third transfer with addition of casein hydrolysate (1 g/l) and glutamine (2 g/l) in an attempt to soften the callus growth. These two ingredients were used in Litvay media - (LM) for gymnosperm somatic embryogenesis (Litvay et al., 1985).

After 4 transfers, the ten lines that had the most growth were tested for embryogenicity. B5 maturation media containing four levels of abscisic acid (ABA) and three levels of sucrose (Table 6.2). Approximately 11.25 g of soft callus material jar (enough to give 3-4 plates of each maturation test media per line) was teased apart and placed into a pre-weighed sterile. A 20% v/w suspension was created by adding 4X the callus weight with B5 hormone-free liquid media. The suspension was placed on an orbital shaker at 125 rpm when not being plated out. A 750 µl sample was pipetted into a Buchner funnel in which sterile filter papers had been placed. The sample was vacuum filtered and the filter paper placed onto petri plates containing 0.8% charcoal media containing 2% sucrose. After two weeks, the filter papers were transferred to maturation test media.

6.3. Results

Though germination hormones were present in the embryo, these appeared to be overpowered by the 2,4-D and BAP and plant growth was limited to approximately 0.5 cm. The area of callusing was variable on germinates, regardless of media or hormone levels. Callusing was observed at root tip, along the stem or on cotyledons - wherever germinates touched the media (Plates 6.1 a and b). Callus growth was extremely slow (Table 6.2).



(a) (b)

Plates 6.1 a and b. Mature seeds producing callus on MS and B5 media.

Table 6.2 . Somatic embryogenesis results.

Media	2,4-D/BAP (μM)	Results
MS	2.3/2.0	1/36 embryos with viable growth/callus production
MS	4.5/2.0	all embryos with no growth/callus production
MS	6.8/2.0	all embryos with no growth/callus production
MS	9.0/2.0	all embryos with no growth/callus production
MS	11.3/2.0	all embryos with no growth/callus production
MS	13.6/2.0	2/36 embryos with viable growth/callus production
B5	2.3/2.0	all embryos with no growth/callus production
B5	4.5/2.0	all embryos with no growth/callus production
B5	6.8/2.0	16/36 embryos with viable growth/callus production
B5	9.0/2.0	8/36 embryos with viable growth/callus production

Table 6.2 continued.

B5	11.3/2.0	10/35 embryos with viable growth/callus production
B5	13.6/2.0	7/36 embryos with viable growth/callus production
MS	2.3/0.21	43 embryos with no viable growth
MS	4.5/0.21	48 embryos with no viable growth
MS	6.8/0.21	33 embryos with no viable growth (1 embryo mouldy)
MS	9.0/0.21	51 embryos with no viable growth
MS	11.3/0.21	44 embryos with no viable growth
MS	2.3/0.62	42 embryos with no viable growth
MS	4.5/0.62	44 embryos with no viable growth
MS	6.8/0.62	45 embryos with no viable growth
MS	9.0/0.62	47 embryos with no viable growth
MS	11.3/0.62	54 embryos with no viable growth

Growth of the maturing cultures was limited with no mature or developing embryos visible (Appendix VI). It is uncertain why the callus material did not grow, but it may be attributed to a lack of auxin in the maturation media.

6.4. Discussion

Previous experiments attempting to obtain embryogenic callus resulted in minimal success. Callus had been grown to approximately 1 cm in diameter but turned brown, became hard and died. Although callus has been grown successfully, the nutrient requirements for the provision of continuous soft callus growth has not been adequately determined. If callus can be cultured continuously, even if it is not embryogenic, it will provide valuable information. Such callus can be manipulated through the use of other hormones to produce shoots and roots.

6.5. Conclusion

While *P. senega* callus has been successfully produced, it was shown to be extremely slow growing and non-embryogenic. Additional combinations of hormones at various levels require

investigation, as do different plant explants. If an auxin is required for callus growth during maturation, it may also be necessary for embryo production.

7. CONCLUSIONS

Considerable progress was achieved for the development of a propagation and plug delivery system for *Polygala Senega*. Perhaps the most outstanding progress was made in the area of seed germination enhancement, where sulphuric acid and potassium hydroxide treatments yielded up to 64% germination. These germination rates are significantly higher than germination rates observed in nature, which are purported to be less than 10%. Sulphuric acid treatments were also implicated as the best method thus far for producing germplasm for embryogenesis trials, though other explants such as apical buds and root callus from micropropagated plants need to be studied.

Notable achievement was also achieved with micropropagation success. The first trial had *in vitro* plant survival, however, poor rooting success resulted in complete mortality when transplanted to a greenhouse. A second trial using a modified MS media had more promising results for *in vitro* rooting. Greenhouse survival will not be known until this spring. Further research is required in order to increase rooting success *in vitro*. Success in this area will have positive implications for subsequent post-transplant survival.

Trials for the production of artificial seeds through embryogenesis techniques resulted in the successful production of callus. The extremely slow rate of growth of the tissue, however, is cause for concern. Future research is required to determine the nutrient requirements for the provision of continuous growth of callus. Future trials will also include immature embryos, micropropagated explants and root crown callus.

In regards to greenhouse husbandry trials, considerable progress was achieved during this project. While survival of transplanted germinates under greenhouse conditions has been lower than desired, the discovery of a successful growth media for greenhouse trials (PGX soiless mix) indicates that the development of a deliverable plug system is well underway. Further tests on plug production will be forthcoming to determine if *P. senega* germinates can survive in a

BX/clay/flax straw plug. Field husbandry trials for *P. senega* indicated that most mature plants harvested from natural sites survive and continue to grow after being transplanted in a sheltered outdoor setting.

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APPENDIX I. General information on *Polygala senega*.
(Information source http://www.botanical.com/botanical/mgmh/s/senega41.html)

Botanical: *Polygala senega* (LINN)

Family: N.O. Polygalaceae

Synonyms: Snake Root, Senegae Radix, Seneca, Seneka, *Polygala virginiana, Plantula marilandica, Senega officinalis,* Milkwort, Mountain Flax, Rattlesnake Root.

Part Used: Dried Root

Habitat: North America

Description

The name of the genus, *Polygala*, means 'much milk', alluding to its own profuse secretions and their effects. 'Senega' is derived from the Seneca tribe of North American Indians, among whom the plant was used as a remedy for snakebites. The plant is a perennial, approximately 6-20 inches tall. The alternate leaves are small and scale-like near the bottom of the stem but lanceolate to ovate and sometimes over an inch wide near the top of the plant. The small greenish-white flowers are contained on a single raceme.

Figure 5. *Polygala senega* growth in natural habitat.

Two black seeds are produced in each capsule. The seeds are 3/16 inch long and have a two-lobed aril-like outgrowth at the micropylar end (see Figure 4). The root, varying in colour from

light yellowish grey to brownish grey, and in size from the thickness of a straw to that of the little finger, has as its distinguishing mark a projecting line, along its concave side. It is usually twisted, sometimes almost spiral, and has at its upper end a thick, irregular, knotty crown just below the soil surface, showing traces of numerous, wiry stems. It breaks with a short fracture, the wood often showing an abnormal appearance, since one or two wedge-shaped portions may be replaced by parenchymatous tissue, as if a segment of wood had been cut out. The keels are due to the development of the bast, and not to any abnormality in the wood. The odour and taste resemble that of Wintergreen.

About 1735, Dr. John Tennent, a Scottish physician living in Pennsylvania, was introduced to the use of P. senega root by the Seneca Indians whom used it for curing rattlesnake bites. As the symptoms were similar to those of pleurisy and the latter stages of pleuropneumonia, he experimented with it in the treatment of those diseases. The root proved beneficial and as a result, the drug was accepted in Europe and cultivated in England in 1739. The roots should be gathered when the leaves are dead, and before the first frost. Due to carelessness in collection other roots are often found mixed with it, but not for intentional adulteration. The root of commerce is also obtained from *Polygala latifolia*, this species being several inches taller and having larger leaves than P. senega. The dried roots, usually in broken pieces, are brought into market in bales weighing from 50 to 400 lb. They vary a little in appearance according to their locality. The official Senega is the small Southern Senega, of which 400 to 500 dried roots are required to make a pound. Manitoba Senega is larger and darker, often with purple markings near the crown. The Northern, White, False, or Large Senega, comes from Wisconsin, Minnesota, and farther west. Approximately 80 to 100 roots are required to make a pound. It is stated that it is not possible to distinguish the two when powdered.

Constituents

The root contains many compounds including polygalic acid, pectin, yellow, bitter, colouring matter, woody fibre, salts, and sugars (see Appendix II). The powder is yellowish-grey to light yellowish-brown. The active principle, contained in the bark, is Senegrin (which some authorities regard as a synonym for polygalic acid, while others differentiate between the two). It is a white powder easily soluble in hot water and alcohol, forming a soapy emulsion when

mixed with boiling water. It is almost identical with the saponin of *Saponaria officinalis* and *Quillaria sapornaria*, a tropical tree found in the Brazilian rain forest. Thus its influence counteracts, or can be counteracted, by digitalis.

Another analysis gives fixed oil, resin, traces of volatile oil (a mixture of valeric ether and methyl salicylate), 7% sugar, from 2-5% senegins, yellow colouring matter, and malates. It is advisable to use an alkali in small proportion in making galenical preparations of senega. Oil of Senega is bitter, rancid, and disagreeable, with the consistency of syrup and an acid reaction. It is not Seneca oil.

Medicinal Action and Uses

It is a stimulating expectorant, diuretic, and diaphoretic. The Ancients regarded its action as identical with that of ipecacuanha, but in doses of three times the strength. It should be used when the power to expectorate is small -- very useful in the second stage of acute bronchial catarrh or pneumonia (www.botanical.com). It is of little value when the expectoration is tough and scanty, but very helpful in chronic pneumonia or bronchitis or dropsy dependent on renal disease. Spirit of chloroform will lessen its disagreeable taste. It has been used also in croup, whooping cough, and rheumatism. As it stimulates most of the secretions, it is also useful as a sialagogue and emmenagogue. In active inflammation its use is contraindicated. In large doses it is emetic and cathartic (www.botanical.com).

Dosages

Powdered root, 5-20 grains. Fluid extract, 10-20 drops. Of infusion, B.P., 4-8 drachms. Of syrup, U.S.P., 1 drachm. Of tincture, B.P., 1/2 - 1 drachm. Conct. Solut., B.P., 1/2 - 1 drachm.

Poisons and Antidotes

In overdose it can act as an irritant or general protoplasmic poison, with violent vomiting and purging. A dose of from 10 minims of the tincture to a scruple of the powdered root will cause heaviness and vertigo, dazzling vision, sneezing, inflammation of the oesophagus, with constriction, thirst, nausea, mucous vomiting, colic, scalding, frothy urine, irritation of the larynx, and general debility. Like saponin, it causes a paresis of the muscles of the respiratory

tract and the vasomotor system in general, resulting in capillary congestions followed by rapid exosmosis.

Appendix II List of chemicals extracted from the root of *Polygala senega*.

(Information source: http://chili.rt66.com/hrbmoore/Constituents/Polygala_senega.txt)

2-O-alpha-d-galactopyranosyl-1, 5-anhydro-d-glucitol

3,4,5-trimethoxycinnamic acid

3,4-dimethoxycinnamic acid

6-O-β-d-glucopyranosyl-1, 5-anhydro-d-glucitol

α-spinasterol arabinose

Arabinosy-α-d-galactosyl-d-glucose

Cyclosenegin

E-senegasaponins A, B & C

Ferulic acid

Fucose

Galactose

Glucose

Hydroxysenegin

Manninotriose

Methyl-salicylate 1,000 ppm

Monotropitoside

p-Coumaric acid

p-Methoxycinnamic acid

Pectin

Polygalic acid

Polygalitol 12,000 ppm

Polygalasaponins I-X

Presenegin

Raffinose

Rhamnose

Ribose

Salicylic acid methyl ester primveroside

Saponins 20,000 -- 100,000 ppm

Senegenic acid

Senegenin

Senegrin

Senegins II & III Senegoses A -- O

Sinapic acid

Stachyose

Tenuifolic acid

Tenuifolin

Tenuigenin

Valerianic Acid

Xylose

z-Senegasaponins A, B & C

z-Senegins I -- IV

Appendix III. Modified Hoagland's solution used in micropropagation

	Initial Modified Hoagland's Solution		Subsequent Modified Hoagland's Solution		Original Hoagland's Solution	
Solution A	g/l in stock solution	Solution Final Concentration	g/l in stock solution	Solution Final Concentration	g/l in stock solution	Solution Final Concentration
$Ca(NO_3)_2 \cdot 4H_2O$	94	3.97 mM/940 ppm	120	5.08 mM/1200 ppm	94	3.97 mM/940 ppm
Solution B						
KNO_3	66	6.52 mM/660 ppm	80	7.91 mM/800 ppm	66	6.52 mM/660 ppm
$MgSO_4 \cdot 7H_2O$	52	2.11 mM/520 ppm	52	2.11 mM/520 ppm	52	2.11 mM/520 ppm
KH_2PO_4	14.2	0.815 mM/142 ppm	14.2	0.815 mM/142 ppm	---	---
Iron Chelate	10	0.125 mM/100 ppm	40	5.01 mM/400 ppm	10	0.125 mM/100 ppm
$NH_4H_2PO_4$	---	---	---	---	12	1.04 mM/120 ppm
Solution C						
H_3BO_3	28	45.3 µM/2.8 ppm	35	56.6 µM/3.5 ppm	28	45.3 µM/2.8 ppm
$MnSO_4 \cdot H_2O$	34	20.1 µM/3.4 ppm	136	80.5 µM/13.6 ppm	34	20.1 µM/3.4 ppm
$CuSO_4 \cdot 5H_2O$	1	0.400 µM/0.1 ppm	28	11.2 µM/2.8 ppm	1	0.400 µM/0.1 ppm
$ZnSO_4 \cdot 7H_2O$	2.2	0.765 µM/0.22 ppm	15.4	5.36 µM/1.54 ppm	2.2	0.765 µM/0.22 ppm
$Na_2MoO_4 \cdot 2H_2O$	0.3	0.124 µM/0.03 ppm	2.4	0.992 µM/0.24 ppm	---	---
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	---	---	---	---	1	0.0809 µM/0.1 ppm

To make 1 litre of modified Hoagland's solution (stir between additions):

-Add 10 ml Solution A
-Fill to 980 ml with water
-Add 10 ml Solution B
-Add 0.1 ml Solution C
-Final pH 6.4 -- 6.7

Appendix IV. MS-based media used in micropropagation

MS Macronutrients 25X Stock	grams/litre	Final concentration in stock solution
NH_4NO_3	41.25	0.5154 M
KNO_3	47.5	0.4698 M
$MgSO_4 \cdot 7H_2O$	9.25	0.03753 M
KH_2PO_4	4.25	0.03123 M
MS Micronutrients 200X Stock		
H_3BO_3	1.24	20.05 mM
$MnSO_4 \cdot H_2O$	3.38	20.00 mM
$ZnSO_4 \cdot 7H_2O$	1.72	5.982 mM
$Na_2MoO_4 \cdot 2H_2O$	0.05	0.2428 mM
$CuSO_4 \cdot 5H_2O$	0.005	0.02003 mM
$CoCl_2 \cdot 6H_2O$	0.005	0.02101 mM
MS Vitamins 200X Stock		
I-Inositol	20.0	0.1110 M
Glycine	0.40	5.333 mM
Nicotinic Acid	0.10	0.8123 mM
Pyridoxine HCl	0.10	0.4864 mM
Thiamine HCl	0.02	0.05929 mM
IBA 100X/1000X Stock	0.10/1.0	0.4144/4.144 mM
Fe (Fe-EDTA 200X stock)	5.56	20.00 mM
$CaCl_2 \cdot 2H_2O$ 500X Stock	220.0	1.496 mM
BAP 1000X Stock	1.0	4.439 mM
KI 1000X Stock	8.3	50.00 mM
2,4-D 1000X Stock	1.0	4.525 mM
NAA 200X Stock	0.2	1.074 mM

Appendix V. Soil and plant analysis of *Polygala senega* taken June – August 2001

Soil Sample Analysis

Site	Texture	pH	EC	NO3-N	P	K	SO4-S	Cu	Fe	Mn	Zn	B	Ca	Mg	Na
A	CL	8.7	0.1	3.0	1.3	140	10	1.28	9.2	4.8	0.38	0.52			
A	CL	8.5	0.1	3.6	<1.0	160	12	1.28	11.0	4.2	0.44	0.54			
A	CL	8.6	0.1	3.2	<1.0	160	13	1.28	8.6	4.2	0.30	0.58			
B	CL	8.4	0.2	3.2	1.1	250	12	1.34	16.6	8.8	0.62	0.88			
B	CL	8.7	0.2	3.6	1.1	150	14	1.10	10.0	5.8	0.32	0.78			
B	CL	8.5	0.2	3.2	<1.0	180	9.8	1.38	28.4	7.2	0.64	0.74			
SB1	SL	7.9	0.6	3.64	1.82	185	>24.1	0.82	22.3	14.5	1.68	1.05	6480	371	260
SB2	L	6.9	0.2	1.36	0.91	217.3	10	0.64	45.5	12.8	0.41	0.59	1180	450	270
PPS1	CL	6.8	0.5	4.4	***>30***	540	>24	0.9	102.0	16.4	6.2	0.3	2300	810	140
PPS2	CL	6.6	0.3	***30.8***	***>30***	550	>24	0.8	86.8	14.4	6.6	0.4	1900	730	140

Note: Electrical conductivity (EC) is in micro Sieverts per centimetre. All nutrients are in parts per million (ppm)

Plant Tissue Analysis of *Polygala seneca*.

Site	Total N %	Total P %	Total K %	Total S %	Total Ca %	Total Mg %	Cu ppm	Fe ppm	Mn ppm	Zn ppm	B ppm
A	2.5	0.24	1.0	0.17	0.31	0.23	10	210	53	26	16
A	2.7	0.30	1.1	0.20	0.64	0.40	18	640	100	30	21
A	2.6	0.25	0.92	0.17	0.48	0.30	16	490	75	31	18
B	2.6	0.28	1.1	0.22	0.46	0.26	12	290	89	31	19
B	2.6	0.26	0.92	0.19	0.42	0.24	11	230	85	29	18
B	3.2	0.28	0.96	0.21	0.40	0.23	11	120	82	30	19

Legend
PPS = Prairie Plant Systems Field Site
Site A =Broadview (confidential)
Site B = Broadview (confidential)
Site SB! = St. Brieux

Appendix VI. Maturation test results.

Line	Sucrose/ABA (μM)	Results
1-1132	90/60	2 plates no growth, 2 plates small amount of growth, no embryos
	180/60	2 plates no growth, 2 plates small amount of growth, no embryos
	270/60	3 plates small amount of growth, 1 plate contaminated, no embryos
	90/120	3 plates small amount of growth
	180/120	4 plates small amount of growth
	270/120	4 plates no growth
	90/180	3 plates no growth, 1 plate contaminated
	180/180	2 plates minimal growth, 1 plate contaminated
	270/180	4 plates no growth
	90/240	4 plates no growth
	180/240	3 plates no growth
	270/240	3 plates minimal growth, 1 plate no growth
1-1027	90/60	4 plates no growth
	180/60	2 plates minimal growth, 1 plate no growth, 1 plate contaminated
	270/60	2 plates no growth, 2 plates contaminated
	90/120	2 plates no growth, 2 plates contaminated
	180/120	2 plates no growth, 2 plates contaminated
	270/120	3 plates no growth, 1 plate minimal growth
	90/180	3 plates no growth
	180/180	4 plates no growth
	270/180	4 plates no growth
	90/240	3 plates no growth
	180/240	2 plates no growth, 2 plates contaminated
	270/240	3 plates no growth, 1 plate contaminated
1-1016	90/60	3 plates minimal growth, 1 plate no growth
	180/60	2 plates no growth, 2 plates contaminated
	270/60	2 plates no growth, 2 plates contaminated
	90/120	2 plates minimal growth, 2 plates contaminated
	180/120	2 plates minimal growth, 2 plates contaminated
	270/120	2 plates minimal growth, 2 plates no growth
	90/180	3 plates no growth, 1 plate contaminated
	180/180	4 plates no growth
	270/180	4 plates minimal growth
	90/240	3 plates no growth, 2 plates contaminated
	180/240	2 plates no growth, 2 plates contaminated
	270/240	3 plates no growth, 1 plate contaminated
1-1120	90/60	2 plates minimal growth, 1 plate no growth, 2 plates contaminated
	180/60	5 plates no growth
	270/60	3 plates minimal growth, 1 plate no growth
	90/120	4 plates no growth
	180/120	4 plates no growth
	270/120	4 plates no growth
	90/180	3 plates no growth
	180/180	3 plates no growth, 1 plate minimal growth, 1 plate contaminated
	270/180	4 plates no growth
	90/240	3 plates no growth, 1 plate contaminated
	180/240	4 plates no growth
	270/240	4 plates no growth

Appendix VI continued.

1-1055	90/60	3 plates minimal growth
	180/60	4 plates minimal growth
	270/60	4 plates minimal growth
	90/120	2 plates minimal growth, 1 plate no growth, 1 plate contaminated
	180/120	4 plates minimal growth
	270/120	2 plates no growth, 1 plate contaminated
	90/180	4 plates no growth
	180/180	4 plates small amount of growth
	270/180	2 plates no growth, 2 plates contaminated
	90/240	2 plates no growth, 2 plates minimal growth
	180/240	1 plate no growth, 2 plates small amt. of growth,1 plate contaminated
	270/240	1 plate no growth, 3 plates small amount of growth
1-1022	90/60	1 plate no growth, 2 plates minimal growth,1 plate contaminated
	180/60	1 plate no growth, 3 plates contaminated
	270/60	4 plates no growth
	90/120	4 plates small amount of growth
	180/120	1 plate no growth, 3 plates contaminated
	270/120	3 plates no growth, 1 plate small amount of growth
	90/180	2 plates small amount of growth, 2 plates contaminated
	180/180	3 plates no growth, 1 plate contaminated
	270/180	4 plates no growth
	90/240	2 plates no growth, 2 plates contaminated
	180/240	3 plates minimal growth, 1 plate contaminated
	270/240	4 plates no growth
1-1029	90/60	4 plates no growth
	180/60	2 plates no growth, 2 plates contaminated
	270/60	3 plates minimal growth,1 plate contaminated
	90/120	3 plates minimal growth,1 plate contaminated
	180/120	3 plates no growth, 1 plate contaminated
	270/120	4 plates no growth
	90/180	4 plates no growth
	180/180	3 plates no growth, 1 plate contaminated
	270/180	3 plates no growth, 1 plate contaminated
	90/240	2 plates no growth, 2 plates contaminated
	180/240	2 plates no growth, 1 plate contaminated
	270/240	3 plates no growth, 1 plate contaminated
1-1068	90/60	2 plates minimal growth, 2 plates no growth
	180/60	4 plates no growth
	270/60	2 plates minimal growth, 2 plates no growth
	90/120	4 plates no growth
	180/120	1 plates minimal growth, 3 plates no growth
	270/120	4 plates no growth
	90/180	3 plates no growth, 1 plate contaminated
	180/180	1 plate minimal growth, 3 plates no growth
	270/180	3 plates no growth, 1 plate contaminated
	90/240	3 plates no growth, 1 plate contaminated
	180/240	1 plate minimal growth, 2 plates no growth, 1 plate contaminated
	270/240	4 plates no growth

Appendix VI continued.

1-1004	90/60	3 plates no growth, 1 plate contaminated
	180/60	2 plates minimal growth, 1 plate no growth, 1 plate contaminated
	270/60	3 plates no growth, 1 plate contaminated
	90/120	3 plates no growth, 1 plate contaminated
	180/120	2 plates minimal growth, 1 plate no growth, 1 plate contaminated
	270/120	4 plates no growth
	90/180	2 plates minimal growth, 2 plates no growth
	180/180	4 plates no growth
	270/180	2 plates no growth, 1 plate contaminated
	90/240	3 plates no growth, 1 plate minimal growth
	180/240	4 plates no growth
	270/240	3 plates no growth
1-1114	90/60	3 plates no growth, 1 plate minimal growth
	180/60	3 plates no growth, 1 plate minimal growth
	270/60	2 plates minimal growth, 2 plates no growth, 1 plate contaminated
	90/120	4 plates minimal growth, 1 plate no growth
	180/120	3 plates no growth
	270/120	4 plates no growth
	90/180	4 plates no growth
	180/180	3 plates minimal growth, 1 plate no growth
	270/180	4 plates no growth
	90/240	4 plates no growth
	180/240	4 plates no growth
	270/240	4 plates no growth

Appendix VII. List of Personnel

Name	Role	Date Worked
Paul Lummerding	Lab Manager	May 2000 – December 2002
Desta Haimanot	Lab Technician	May 2000 – December 2002
Jerilyn Hamm	Lab/Greenhouse Technician	May 2000 – December 2001
Ping Ng	Lab/Greenhouse Technician	May 2000 – December 2001
Debora Verbonac	Lab/Greenhouse Technician	November 2001- September 2002
Jackie Masich	Greenhouse Manager	May 2000 – June 2002
Mary Bertoncini	Assistant Greenhouse Manager	May -Dec 2000/Jan – Dec 2002
Tasha Bedry	Greenhouse Technician	May 2000 – December 2001
Kelly Busch	Greenhouse Technician	May – December 2000
Maureen Letkeman	Greenhouse Technician	May 2000 – December 2002
Natasha Lomond	Greenhouse Technician	May – December 2000
Leon Perehudoff	Greenhouse Technician	May – December 2000
Rahmatullah Shoghi	Greenhouse Technician	May 2000 – December 2002
Kim Stevenson	Greenhouse Technician	May – December 2000
Stacy Strom	Greenhouse Technician	May 2000 – December 2001
Mark Hetherington	Senior Research Officer	May 2000 – December 2002
Mark Schierling	Greenhouse Technician	May 2000 – December 2002
Erin Hupaelo	Greenhouse Technician	May – August 2001/2002
Dion Walker	Greenhouse Technician	May – August 2001/2002
Susan Dormer	Greenhouse Technician	May 2000 – December 2001
Brenda Jackson	Greenhouse Technician	May – September 2001
Rilla Yaschuk	Greenhouse Technician	May – Sept 2001/2002
Brent Zettl	Project Administrator & Overall Management	May 2000 – December 2002